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**(54) Title:** METHODS FOR GENETICALLY MODIFYING HEMATOPOIETIC STEM CELLS

**(57) Abstract**

The invention provides methods of genetically modifying hematopoietic stem cells using viral vectors bearing the vesicular stomatitis virus G-glycoprotein.

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# METHOD FOR GENETICALLY MODIFYING HEMATOPOIETIC STEM CELLS

## INTRODUCTION

### Technical Field
The field of this invention is methods for genetic
modification of hematopoietic stem cells.

### Background
Mammalian hematopoietic cells provide a diverse range of
physiological activities.  These cells are divided into
lymphoid, myeloid and erythroid lineages.  The lymphoid
lineages, comprising B cells and T cells, provides for the
production of antibodies, regulation of the cellular immune
system, detection of foreign agents in the blood, detection
of cells foreign to the host, and the like.  The myeloid
lineage, which includes monocytes, granulocytes,
megakaryocytes as well as other cells, monitors for the
presence of foreign bodies, provides protection against
neoplastic cells, scavenges foreign materials, produces
platelets, and the like.  The erythroid lineage provides the
red blood cells, which act as oxygen carriers.
All publications cited herein are hereby incorporated herein
by reference in their entirety.
Despite the diversity of the nature, morphology,
characteristics and function of hematopoietic cells, it is
presently believed that these cells are derived from a single
progenitor population, termed "stem cells."  Stem cells are
capable of self-regeneration and may become lineage committed
progenitors which are dedicated to differentiation and
expansion into a specific lineage.  As used herein, "stem
cells" refers to hematopoietic cells and not stem cells of
other cell types.  Further, unless indicated otherwise, "stem
cells" refers to human hematopoietic stem cells.  U.S. Patent
No. 5,061,620 describes a substantially homogeneous stem cell

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composition and the manner of obtaining such a composition.
See also the references cited therein.

Stem cells constitute only a small percentage of the total
number of hematopoietic cells.  Hematopoietic cells are
identifiable by the presence of a variety of cell surface
"markers."  Such markers may be either specific to a
particular lineage or progenitor cell or be present on more
than one cell type.  Currently, it is not known how many of
the markers associated with differentiated cells are also
present on stem cells.  One marker, which was previously
indicated as present solely on stem cells, CD34, is also
found on a significant number of lineage committed
progenitors.  U.S. Pat. No. 4,714,680 describes a population
of cells expressing the CD34 marker.

Table 1 summarizes probable phenotypes of stem
cells in fetal, adult, and mobilized peripheral blood.  In
Table 1 myelomonocytic stands for myelomonocytic associated
markers, NK stands for natural killer cells and AMPB stands
for adult mobilized peripheral blood.  As used herein both
infra, supra and in Table 1, the negative sign or, uppercase
negative sign, ($^-$) means that the level of the specified
marker is undetectable above Ig isotype controls by FACS
analysis, and includes cells with very low expression of the
specified marker.

**Table 1**

| | Probable Stem Cell Phenotypes | | | | | | | | | | | | | | | | |
| | NK and T cell markers | | | B cell markers | | | Myelomonocytic | | | | Other | | | | | | P-gp Activity |
| | CD2 | CD3 | CD8 | CD10 | CD19 | CD20 | CD14 | CD15 | CD16 | CD33 | CD34 | CD38 | HLA-DR | C-Kit | Thy | Rho | |
| FBM | - | - | - | - | - | - | - | - | - | ? | + | - | + | + | + | lo | + |
| ABM | - | - | - | - | - | - | - | - | - | - | + | ? | lo/- | + | + | lo | + |
| AMPB | - | - | - | - | - | - | - | - | - | lo/-? | + | ? | lo/- | ? | + | lo | + |

The ability of stem cells to undergo substantial self-renewal as well as the ability to proliferate and differentiate into all of the hematopoietic lineages makes stem cells the target of choice for a number of gene therapy applications. Successful gene transfer into stem cells should provide long-term repopulation of an individual with the modified cells and their progeny, which will express the desired gene product. By contrast, gene transfer into more mature hematopoietic cells, such as T cells, at best, provides only transient therapeutic benefit. Thus, there have been world-wide efforts toward finding effective methods of genetically modifying stem cells. For reviews of genetic modification of stem cells see Brenner (1993) *J. Hematother.* 2:7-17; and Moore and Belmont.

Most efforts to genetically modify stem cells have involved the use of retroviral vectors. Other methods such as liposome-mediated gene transfer or adeno-associated viral vectors have also been used. Retroviral vectors have been the primary vehicle due to the generally high rate of gene transfer obtained in experiments with cell lines, and the ability to obtain stable integration of the genetic material, which ensures that the progeny of the modified cell will contain the transferred genetic material. Retroviral vectors and their use in the transfer and expression of foreign genes are reviewed in Gilboa (1988) *Adv. Exp. Med. Biol. 241*:29; Luskey et al. (1990) *Ann. N.Y. Acad. Sci. 612*:398; and Smith (1992) *J. Hematother. 1*:155-166.

Currently used methods of retroviral transduction into human stem cells have a number of practical limitations. One limitation is the extremely low numbers of stem cells present in any tissue. Therefore, in transductions performed with relatively impure populations of cells, the ratio of virus particles to stem cells will be quite low. This limitation is compounded by the relatively low titers generally obtained with most retroviral vectors, typically in the range of $10^5$ to $10^6$ infectious virions per milliliter.

        In addition, primitive stem cells typically are
quiescent in culture; retroviral vectors require target cells
to be cycling for stable integration of the retroviral DNA.
Cytokines may be used to cause stem cells to cycle; however,
the effect of various cytokines in driving stem cells to
differentiation remains in question.  Likewise, the effect of
more differentiated cells in culture on the growth or
division of stem cells is not well understood.

        Many retroviral vectors currently in use are
derived from the Moloney murine leukemia virus (MoMLV).  In
most cases, the viral gag, pol and env sequences are removed
from the virus, creating room for insertion of foreign DNA
sequences.  Genes encoded by the foreign DNA are usually
expressed under the control of the strong viral promoter in
the LTR.  Such a construct can be packed into viral particles
efficiently if the gag, pol and env functions are provided in
trans by a packaging cell line.  Thus, when the vector
construct is introduced into the packaging cell, the gag-pol
and env proteins produced by the cell, assemble with the
vector RNA to produce infectious virions that are secreted
into the culture medium.  The virus thus produced can infect
and integrate into the DNA of the target cell, but does not
produce infectious viral particles since it is lacking
essential packaging sequences.  Most of the packaging cell
lines currently in use have been transfected with separate
plasmids, each containing one of the necessary coding
sequences, so that multiple recombination events are
necessary before a replication competent virus can be
produced.  Alternatively, the packaging cell line harbors an
integrated provirus.  The provirus has been crippled so that,
although it produces all the proteins required to assemble
infectious viruses, its own RNA cannot be packaged into
virus.  Instead, RNA produced from the recombinant virus is
packaged.  The virus stock released from the packaging cells
thus contains only recombinant virus.

        The range of host cells that may be infected by a

retrovirus or retroviral vector is determined by the viral
envelope protein.  The recombinant virus can be used to
infect virtually any other cell type recognized by the env
protein provided by the packaging cell, resulting in the
integration of the viral genome in the transduced cell and
the stable production of the foreign gene product.  The
efficiency of infection is also related to the level of
expression of the receptor on the target cell.  In general,
murine ecotropic env of MoMLV allows infection of rodent
cells, whereas amphotropic env allows infection of rodent,
avian and some primate cells, including human cells.
Amphotropic packaging cell lines for use with MoMLV systems
are known in the art and include, but are not limited to,
ΨAM, PA12, PA317, and ΨCRIP.  Miller et al. (1985) *Mol. Cell.
Biol. 5*:431-437; Miller et al. (1986) *Mol. Cell. Biol.
6*:2895-2902; and Danos et al. (1988) *Proc. Natl. Acad. Sci.
USA 85*:6460-6464.  Xenotropic vector systems exist which also
allow infection of human cells.

The host range of retroviral vectors has been
altered by substituting the env protein of the base virus
with that of a second virus.  The resulting, "pseudotyped",
virus has the host range of the virus donating the envelope
protein and expressed by the packaging cell line.  Recently,
the G-glycoprotein from vesicular stomatitis virus (VSV-G)
has been substituted for the MoMLV env protein.  Burns et al.
(1993) *Proc. Natl. Acad. Sci USA 90*:8033-8037; and PCT patent
application WO 92/14829.  Unlike typical amphotropic env
proteins, the VSV-G protein is thought to mediate viral
infection by fusing with a phospholipid component of cell
membranes rather than by recognition of a cell surface
protein.  Since infection is not dependent on a specific
receptor, VSV-G pseudotyped vectors have a broad host range.
Indeed, MoMLV-based retroviral vectors pseudotyped with VSV-G
were shown to have a broad host range, infecting a number of
cell lines derived from species such as hamster and fish.
These types of cell lines are not normally infected by

viruses containing the MoMLV envelope protein.

The mechanism of infection mediated by the VSV-G protein, however, has not been elucidated. It has been reported that VSV-G interacts with a phosphatidylserine component of cell membranes, but that observation has yet to be verified. Schlegal et al. (1983) *Cell 32*:639-646. As a result, it cannot be predicted with any certainty what cells or cell types would be infected by a VSV-G pseudotyped vector, or with what efficiency transduction might occur.

## Summary of the Invention

The invention provides improved methods of transducing hematopoietic stem cells with pseudotyped retrovirus vectors containing the VSV-G protein. The methods comprise using the vectors to transduce a population of hematopoietic cells enriched for stem cells.

## Brief Description of the Drawings

Figure 1 is a schematic depiction of the plasmid pME-VSV-G.

Figure 2 is a schematic depiction of the plasmid MFG-lac-Z.

## Description of the Specific Embodiments

It has now been found that CD34$^+$Thy-1$^+$ mobilized peripheral blood (MPB) cells are transduced with surprisingly high efficiency by a VSV-G pseudotyped retroviral vector as compared to CD34$^+$ adult bone marrow (ABM) cells and as compared to the transduction efficiency of a conventional amphotropic vector. The improvement appears to be specific to the vector and cell type; an amphotropic vector containing the same viral genome showed a somewhat better transduction efficiency in CD34$^+$ ABM cells compared to CD34$^+$Thy-1$^+$ MPB cells.

While not intending to be bound by any one theory, the improved transduction efficiency described herein appears

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to be due either to the more purified stem cell population, represented by CD34$^+$Thy-1$^+$Lin$^-$ cells as compared to CD34$^+$ cells, or due to the tissue cell source. Stem cells that have been mobilized into the peripheral blood by chemotherapy and/or cytokines may undergo changes in membrane characteristics as a result of the activation or mobilization; these changes may affect binding by VSV-G. Therefore, less highly purified stem cells obtained from MPB may also be suitable for use herein. Alternatively, more primitive hematopoietic cells represented by CD34$^+$Thy-1$^+$Lin$^-$ cells may be preferentially infected by the pseudotyped vector regardless of tissue source. In addition, primitive cells may cycle more when purified from more mature progenitor cells, thereby increasing integration of the retroviral genome. In that case, it is likely that any retroviral vector will transduce pluripotent stem cells more efficiently when a purer stem cell population is transduced as compared to the more heterogenous CD34$^+$ cell population. In the presence of less primitive cells, stem cells will cycle less causing transduction frequency to represent disproportionate transduction of progenitor cells at the expense of stem cells.

As used herein, "stem cells" refers to a population of hematopoietic cells more highly enriched in pluripotent stem cells than the population characterized solely by CD34 expression. In particular, "stem cells" refers to a subpopulation of CD34$^+$ cells having all of the long-term engrafting potential *in vivo*. Animal models for long-term engrafting potential include the SCID-hu bone model and the *in utero* sheep model. For review, see Srour et al. (1992) *J. Hematother.* *1*:143-153 and the references cited therein. In assays for long-term culture-initiating cells (LTCIC), the highly enriched stem cell population will typically have a LTCIC frequency in the range of 1/20 to 1/100; preferably it will have a frequency of at least 1/50. Stem cells are exemplified by CD34$^+$Thy-1$^+$ MPB cells and described more fully

in the examples provided herein.  Other markers that have
been reported to subdivide CD34⁺ cells, further enriching for
stem cells, include, but are not limited to, CD38⁻;
rhodamine^lo; c-kit receptor⁺; HLADR^lo/⁻; CD71⁻; and CD45RA⁻.
See Table 1.  In fetal tissues and umbilical cord blood, stem
cells are highly enriched in the CD34^hiLin⁻ population as
described by DiGiusto et al. (1993) *Blood* 84:421-432.

The highly enriched populations of stem cells may
be transduced immediately after purification or maintained in
long-term cultures and expanded in number in appropriate
media, optionally in conjunction with hematopoietic factors
such as LIF, stem cell factor, IL3, IL6, IL7, IL11, GCSF,
GMCSF, EPO, MIP-1α and IFNγ, under otherwise conventional
conditions.

Genetic modification of the stem cells can be
accomplished at any point during their maintenance by
transduction with a recombinant DNA construct packaged in a
virion containing the VSV-G protein.  The resulting
transduced cells may then be grown under conditions similar
to those for unmodified stem cells, whereby the modified stem
cells may be expanded and used for a variety of purposes.

Stem cells may be isolated from any known source of
stem cells, including, but not limited to, bone marrow, both
adult and fetal, mobilized peripheral blood (MPB) and
umbilical cord blood.  Initially, bone marrow cells may be
obtained from a source of bone marrow, including but not
limited to, ilium (e.g. from the hip bone via the iliac
crest), tibia, femora, spine, or other bone cavities.  Other
sources of stem cells include, but are not limited to,
embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate
solution may be used to flush the bone, including, but not
limited to, salt solution, conveniently supplemented with
fetal calf serum (FCS) or other naturally occurring factors,
in conjunction with an acceptable buffer at low
concentration, generally from about 5-25 mM.  Convenient

buffers include, but are not limited to, HEPES, phosphate
buffers and lactate buffers.  Otherwise bone marrow may be
aspirated from the bone in accordance with conventional
techniques.

Methods for mobilizing stem cells into the
peripheral blood are known in the art and generally involve
treatment with chemotherapeutic drugs, cytokines (e.g. GM-
CSF, G-CSF or IL3), or combinations thereof.  Typically,
apheresis for total white cells begins when the total white
cell count reaches 500-2000 cells/µl and the platelet count
reaches 50,000/µl.

Various techniques may be employed to separate the
cells by initially removing cells of dedicated lineage
("lineage-specific" cells).  Monoclonal antibodies are
particularly useful for identifying markers associated with
particular cell lineages and/or stages of differentiation.
The antibodies may be attached to a solid support to allow
for crude separation.  The separation techniques employed
should maximize the retention of viability of the fraction to
be collected.  Various techniques of different efficacy may
be employed to obtain "relatively crude" separations.  Such
separations are where up to 10%, usually not more than about
5%, preferably not more than about 1%, of the total cells
present not having the marker may remain with the cell
population to be retained.  The particular technique employed
will depend upon efficiency of separation, associated
cytotoxicity, ease and speed of performance, and necessity
for sophisticated equipment and/or technical skill.

The use of separation techniques include, but are
not limited to, those based on differences in physical
(density gradient centrifugation and counter-flow centrifugal
elutriation), cell surface (lectin and antibody affinity),
and vital staining properties (mitochondria-binding dye
rho123 and DNA-binding dye Hoechst 33342).  Procedures for
separation may include, but are not limited to, magnetic
separation, using antibody-coated magnetic beads, affinity

chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique. Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

A large proportion of the differentiated cells may be removed by initially using a relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation may be employed to remove erythrocytes, by employing Ficoll-Hypaque separation.

The gross separation may be achieved using methods known in the art including, but not limited to, magnetic beads, cytotoxic agents, affinity chromatography or panning. Antibodies which find use include antibodies to lineage specific markers which allow for removal of most, if not all, mature cells, while being absent on stem cells.

Concomitantly or subsequent to a gross separation, which provides for positive selection, a negative selection may be carried out, where antibodies to lineage-specific markers present on dedicated cells are employed. For the most part, these markers include, but are not limited to, $CD2^-$, $CD3^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD14^-$, $CD15^-$, $CD16^-$, $CD19^-$, $CD20^-$, $CD33^-$ and glycophorin A; preferably including, but not limited to, at least $CD2^-$, $CD14^-$, $CD15^-$, $CD16^-$, $CD19^-$ and glycophorin A; and normally including at least $CD14^-$ and $CD15^-$. See Table 1. As used herein, $Lin^-$ refers to a cell population lacking at least one lineage-specific marker. The

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hematopoietic cell composition substantially depleted of
dedicated cells may then be further separated using a marker
for Thy-1, whereby a substantially homogeneous stem cell
population is achieved.  Exemplary of this stem cell
population is a population which is $CD34^+Thy-1^+Lin^-$, which
provides an enriched stem cell composition.

The purified stem cells have low side scatter and
low to medium forward scatter profiles by FACS analysis.
Cytospin preparations show the enriched stem cells to have a
size between mature lymphoid cells and mature granulocytes.
Cells may be selected based on light-scatter properties as
well as their expression of various cell surface antigens.

While it is believed that the particular order of
separation is not critical to this invention, the order
indicated is preferred.  Preferably, cells are initially
separated by a coarse separation, followed by a fine
separation, with positive selection of a marker associated
with stem cells and negative selection for markers associated
with lineage committed cells.

Compositions highly enriched in stem cells may be
achieved in this manner.  The desired stem cells are
exemplified by a population with the $CD34^+Thy-1^+Lin^-$ phenotype
and being able to provide for cell regeneration and
development of members of all of the various hematopoietic
lineages.

It should be noted that negative selection lineage
selection for lineage specific markers provide a greater
enrichment in stem cells obtained from bone marrow than from
MPB.  The majority of CD34. cells that are mobilized into the
peripheral blood do not express lineage-specific markers and,
therefore, Lin. selection does not significantly enrich over
CD34. selection in the peripheral blood as it does in bone
marrow.  Selection for Thy-1. does enrich for stem cells in
both mobilized peripheral blood and bone marrow.

A stem cell composition is characterized by being

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able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and higher order cultures, and being capable of differentiating into the various lymphocytic and myelomonocytic lineages, particularly B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

The stem cells may be grown in culture in an appropriate nutrient medium, including, but not limited to, conditioned medium, a co-culture with an appropriate stromal cell line, adhesion molecules, or a medium comprising a synthetic combination of growth factors which are sufficient to maintain the growth of hematopoietic cells.

For conditioned media or co-cultures, various stromal cell lines may be used. Since human stromal cell lines are not required, other stromal cell lines may be employed, including but not limited to rodentiae, particularly murine. Suitable murine stromal cell lines include AC3 and AC6, which are described in Whitlock et al. (1987) Cell 48:1009-1021. Other stromal cell lines may be developed, if desired. Preferably, the stromal cell line used is a passage of AC6, AC6.21 (otherwise referred to as SyS1).

Various devices exist for co-culture of stem cells with stromal cells which allow for growth and maintenance of stem cells. These include devices employing mechanisms including, but not limited to, crossed threads, membranes and controlled medium flow. These may be employed for the growth of the cells for removal of waste products, and replenishment of the various factors associated with cell growth.

Conveniently, tissue culture plates or flasks may be employed where confluent stromal cell layers may be maintained for extended periods of time without passage, but with changing of the tissue culture medium about every five to seven days.

The stem cells may be grown in co-culture by

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placing the stem cells onto the stromal cell lines, either
directly or separated by a porous membrane.  For example,
about $3 \times 10^4$ to $3 \times 10^5$ cells/ml are placed on a confluent
stromal cell layer.  The media employed in the co-culture may
be any convenient growth medium, including, but not limited
to, RPMI-1640 and IMDM either individually or in combination,
where appropriate antibiotics to prevent bacterial growth,
e.g. penicillin, streptomycin (pen/strep) and other
additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM),
2-mercaptoethanol ($1-10 \times 10^{-5}$ M, 2-ME) and from about 5-15%,
preferably about 10% of serum, e.g. FCS.  Cytokines may also
be added, including, but not limited to, LIF, interleukins,
colony stimulating factors, steel factor.  Of particular
interest are LIF, steel factor, IL-3, IL-6, GM-CSF, G-CSF and
MIP-1$\alpha$.

The factors which are employed may be naturally
occurring or synthetic, e.g. prepared recombinantly, and may
be human or of other species, e.g. murine, preferably human.
The amount of the factors will generally be in the range of
about 1 ng/ml to 100 ng/ml.  Generally, for LIF, the
concentration will be in the range of about 1 ng/ml to 100
ng/mg, more usually 5 ng/ml to 30 ng/ml; for IL-3, the
concentration will be in the range of about 5 ng/ml to 50
ng/ml, more usually 5 ng/ml to 100 ng/ml; for IL-6, the
concentration will be in the range of about 5 ng/ml to 50
ng/ml, more usually 5 ng/ml to 20 ng/ml, and for GM-CSF, the
concentration will generally be 5 ng/ml to 50 ng/ml, more
usually 5 ng/ml to 20 ng/ml.

In one embodiment, the stem cells are optionally
expanded prior to or after transduction.  During expansion,
the growth factors may be present only during the initial
course of the stem cell growth and expansion, usually at
least 24 hours, more usually at least about 48 hours to 4
days or may be maintained during the course of the expansion.

For use in clinical settings, it is preferable to
transduce the stem cells without prior or subsequent

expansion. In one embodiment therefore, the stem cells are cultured with or without cytokines in an appropriate medium, transduced with the appropriate vector, cultured for approximately 72 hours and reintroduced into the host.

Gene transfer into stem cells may be used to treat a variety of neoplastic, infectious or genetic diseases. For example, one may introduce genes that confer resistance to chemotherapeutic agents, thereby protecting the progeny hematopoietic cells, allowing higher doses of chemotherapy and thereby improving the therapeutic benefit. For instance, the mdr1 gene may be introduced into stem cells to provide increased resistance to a wide variety of drugs including taxol, which are exported by the mdr1 gene product, in combination with the administration of chemotherapeutics such as taxol, e.g. for breast cancer treatment. Similarly, genes that provide increased resistance to alkylating agents, such as melphalan, may be introduced into stem cells in conjunction with high dose chemotherapy. Genes that provide resistance to alkylating agents include, but are not limited to, glutathione-S-transferase and methylpurine DNA glycosylate. Other potential drug-resistance genes that could be introduced into stem cells in combination with high-dose chemotherapy include, but are not limited to: adenosine deaminase (ADA) for resistance to purine analogs; O6-alkylguanine-DNA alkyltransferase for resistance to N-methyl-N-nitrosurea; methylguanine methyl transferase for resistance to nitrosurea BCNU; dihydrofolate reductase for resistance to methotrexate; methylpurine DNA glycosylate for resistance to radiation; and glutacyanase transferase for resistance to platinum analogs.

For viral infections that primarily affect hematolymphoid cells, stem cells may be modified to endow the progeny with resistance to the infectious agent. In the case of human immunodeficiency virus (HIV), for example, specific antisense or ribozyme sequences may be introduced that interfere with viral infection or replication in the target

cells.  Alternatively, the introduced gene products may serve as "decoys" by binding essential viral proteins, thereby interfering with the normal viral life cycle and inhibiting replication.  Introduction of the apoptosis modulating genes such as bcr-abl into stem cells, may provide resistance to the cell death by apoptosis associated with HIV infection.

Alternatively, stem cells may be modified to produce a product to correct a genetic deficiency, or where the host has acquired a genetic deficiency through a subsequent disease.  Genes that may correct a genetic deficiency include, but are not limited to, adenosine deaminase for the treatment of ADA⁻ severe combined immunodeficiency; glucocerebrosidase for the treatment of Gaucher's disease; beta-globin for the treatment of sickle cell anemia; and Factor VIII or Factor IX for the treatment of hemophilia.

Suitable viral constructs are discussed in Burns et al. (1993) and PCT application no. WO 92/14829 although any functional pseudotype virus containing the VSV-G gene and capable of being packaged by a packaging cell line is suitable for use herein.  The viral constructs employed will normally include the VSV-G gene, the foreign gene(s) and a marker gene, which allows for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct.  Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin.  Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections could be accomplished by employment of a stable cell surface marker to select for transgene expressing stem cells by FACS sorting.

The viral constructs can be prepared in a variety of conventional ways.  Numerous vectors are now available which provide the desired features, such as long terminal

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repeats, marker genes, and restriction sites, which may be
further modified by techniques known in the art.  The
constructs may encode a signal peptide sequence to ensure
that genes encoding cell surface or secreted proteins are
properly processed post-translationally and expressed on the
cell surface if appropriate.  Preferably, the foreign gene(s)
is under the control of a cell specific promoter as discussed
below.

      In those instances where a particular polymorphic
region of a polymorphic protein, such as a T-cell receptor,
major histocompatibility complex antigen, or immunoglobulin
subunit is involved with susceptibility to a particular
disease, for example an autoimmune disease, the particular
exon may be "knocked out" by homologous recombination, so as
to provide hematopoietic cells which will not be responsive
to the disease.

      Expression of the transferred gene can be
controlled in a variety of ways depending on the purpose of
gene transfer and the desired effect.  Thus, the introduced
gene may be put under the control of a promoter that will
cause the gene to be expressed constitutively, only under
specific physiologic conditions, or in particular cell types.
Examples of promoters that may be used to cause expression of
the introduced sequence in specific cell types include
Granzyme A for expression in T-cells and NK cells, the CD34
promoter for expression in stem and progenitor cells, the CD8
promoter for expression in cytotoxic T-cells, and the CD11b
promoter for expression in myeloid cells.  Inducible
promoters may be used for gene expression under certain
physiologic conditions.  For example, an electrophile
response element may be used to induce expression of a
chemoresistance gene in response to electrophilic molecules.
The therapeutic benefit may be further increased by targeting
the gene product to the appropriate cellular location, for
example the nucleus, by attaching the appropriate localizing
sequences.

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In addition, by appropriate use of inducible promoters, expression of various protein products can be achieved at selected levels of differentiation or in selected cell lineages, or even in response to particular chemicals, such as chemoattractants, particular ligands, and the like. Also, as there is better understanding of the manner in which stem cells differentiate to specific lineages, particular lineages, such as megakaryocytes, subsets of T cells, monocytes, and the like can be produced in culture.

Possible methods of transduction include, but are not limited to, direct co-culture of stem cells with producer cells e.g. by the method of Bregni et al. (1992) Blood 80:1418-1422 or culturing with viral supernatant alone, with or without appropriate growth factors and polycations, e.g. by the method of Xu et al. (1994) Exp. Hemat. 22:223-230; and Hughes et al. (1992) J. Clin. Invest. 89:1817.

In many situations, cell immunotherapy involves removal of bone marrow or other source of stem cells from a human host, isolating the stem cells from the source and optionally expanding the stem cells. Meanwhile, the host may be treated to partially, substantially or completely ablate native hematopoietic capability. The isolated stem cells may be modified during this period of time, so as to provide for stem cells having the desired genetic modification. After completion of the treatment of the host, the modified stem cells may then be restored to the host to provide for expression of the foreign gene. The methods of stem cell removal, host ablation and stem cell repopulation are known in the art. If necessary, the process may be repeated to ensure substantial repopulation of the modified stem cells.

To ensure that the stem cells have been successfully modified, a vector-specific probe may be used to verify the presence of the vector in the transduced stem cells or their progeny. In addition, the cells may be grown under various conditions to ensure that they are capable of maturation to all of the hematopoietic lineages while

maintaining the capability, as appropriate, of the introduced
DNA. Various tests *in vitro* and *in vivo* may be employed to
ensure that the pluripotent capability of the stem cells has
been maintained.

The compositions comprising stem cells provide for
production of myeloid cells and lymphoid cells in appropriate
cultures, cultures providing hydrocortisone for production of
myeloid cells (associated with Dexter-type cultures) and B
lymphocytes in cultures lacking hydrocortisone, (associated
with Whitlock-Witte type cultures). In each of the cultures,
mouse or human stromal cells are provided, which may come
from various sources, including, but not limited to, AC3, AC6
or stromal cells derived from mouse or human FBM by selection
for the ability to maintain stem cells, and the like.
Preferably, the stromal cells are AC6.21 and the ability to
produce B lymphocytes and myeloid cells is determined in
cultures supplied with LIF and IL-6. The stem cells give
rise to B cells, T cells and myelomonocytic cells in the *in
vivo* assays described below.

To demonstrate differentiation to T cells, fetal
thymus is isolated and cultured from 4-7 days at about 25°C,
so as to deplete substantially the lymphoid population. The
cells to be tested for T cell activity are then microinjected
into the thymus tissue, where the HLA of the population which
is injected is mismatched with the HLA of the thymus cells.
The thymus tissue may then be transplanted into a scid/scid
mouse as described in US Patent No. 5,147,784, particularly
transplanting under the kidney capsule.

Specifically, the population of stem cells can be
microinjected into HLA mismatched thymus fragments. After 6-
10 weeks, assays of the thymus fragments injected with stem
cells can be performed and assessed for donor- derived T
cells. Injected thymus fragments injected with cells having
T cell differentiative capacity will generate and sustain
CD3⁺, CD4⁺, and CD8⁺ T cells along with their progenitors.

Further demonstration of the sustained regenerative

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ability of stem cell populations may be accomplished by the detection of continued myeloid and B-lymphoid cell production in the SCID-hu bone model.  Kyoizumi et al. (1992) Blood 79:1704.  To analyze this, one may isolate human fetal bone and transfer a longitudinally sliced portion of this bone into the mammary fat pad of a scid/scid animal.  The bone cavity is diminished in endogenous cells by whole body irradiation of the mouse host prior to injection of the test donor population.  The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells.  Stem cells from human hematopoietic sources sustain B lymphopoiesis and myelopoiesis in the SCID-hu bone model.

For RBCs, one may use conventional techniques to identify BFU-E units, for example methylcellulose culture demonstrating that the cells are capable of developing the erythroid lineage.  Metcalf (1977) In:  Recent Results in Cancer Research 61.  Springer-Verlag, Berlin, pp. 1-227.

A pluripotent stem cell may be defined as follows: (1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised human host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell by self renewal.

The following examples are provided to illustrate but not limit the invention.


Example 1

Cell sorting and analysis


Apheresed samples were obtained with informed consent from multiple myeloma patients treated at the University of Arkansas Medical Center.  The patients were treated on day 1 with cyclophosphamide at 6 g/m$^2$ (1.5 g/m$^2$ every 3 hrs x 4 doses).  From day 1 until the start of leukopheresis (usually 10-28 days), granulocyte macrophage colony stimulating factor (GM-CSF) was given at 0.25

mg/m$^2$/day. Apheresis for total white cells was started when
the peripheral blood white cell count was greater than 500
cells/µl and the platelet count was greater than 50,000
cells/µl. Patients were apheresed daily until from 6 x 10$^8$
mononuclear cells (MNC) were collected.

Antibodies to CD2, CD14, CD15, CD16 and CD19 were
obtained as FITC conjugates from Becton-Dickinson. Antibody
to Thy-1 (GM201) was obtained from Dr. Wolfgang Rettig
(Ludwig Institute, New York), and was detected with anti-γ1-
PE conjugate from Caltag. Antibody to CD34 (Tük 3) was
obtained from Dr. Andreas Ziegler (University of Berlin), and
detected with an anti-γ3-Texas Red conjugate (Southern
Biotechnologies). The antibody to glycophorin A was obtained
as a FITC conjugate from AMAC. The lineage cocktail was a
combination of antibodies to CD2, CD14, CD15, CD16, CD19 and
glycophorin A.

For cell sorting, fresh MPB samples were elutriated
with a JE5.0 Beckman counterflow elutriator equipped with a
Sanderson chamber (Beckman, Palo Alto, CA). Cells were
resuspended in elutriation medium (Biowhittaker,
Walkersville, MD) at pH 7.2, supplemented with 0.5% human
serum albumin (HSA). The rotor speed was set at 2000 RPM,
the cells were introduced, and the first fraction collected
at a flow rate of 9.6 ml/min. Fractions 2 and 3 were
collected at the respective flow rates of 14 and 16 ml/min.
The larger cells remaining in the chamber were collected
after stopping the rotor. Cells were resuspended in RPMI
supplemented with 5% HSA, 10 µg/ml DNAse I and
penicillin/streptomycin at 50 U/ml and 50 µg/ml,
respectively. Fractions 2 and 3 were pooled and incubated
with 1 mg/ml heat-inactivated human gamma-globulin to block
non-specific Fc binding. Granulocytes were further depleted
by incubation with CD15 conjugated to magnetic beads (Dynal
M450, Oslo, Norway) followed by magnetic selection.

Anti-CD34 antibody or an IgG3 isotype matched
control were added to cells in staining buffer (HBSS, 2% FCS,

10 mM HEPES) for 20 minutes on ice, together with anti-Thy-1
antibody at 5 µg/ml. Cells were washed with a FCS underlay,
and then incubated with Texas Red conjugated goat anti-mouse
IgG3 antibody and phycoerythrin-conjugated goat anti-mouse
IgG1 antibody for 20 minutes on ice. Blocking IgG1 was then
added for 10 minutes. After blocking, the FITC-conjugated
lineage antibody panel was added, and incubated for another
20 minutes on ice. After a final washing, cells were
resuspended in staining buffer containing propidium iodide
(PI).

Cells were sorted in the FACSTAR Plus cell sorter
equipped with dual argon ion lasers, the primary laser
emitting at 488 nm and a dye laser (Rhodamine 6G) emitting at
600 nm (Coherent Innova 90, Santa Cruz, CA). Residual
erythrocytes, debris and dead cells were excluded by light
scatter gating plus an FL3 (PI) low gate. Following
isolation of a cell population by FACS, the sample was
diluted 1:1 in HBSS, pelleted, and resuspended in HBSS for
hemocytometer counting.

CD34$^+$ cells were positively selected from cadaveric
adult bone marrow (ABM) using a biotinylated anti-CD34
antibody (K6.1) and a biotin competition release according to
the method described in PCT patent application no.
WO94/02016.

### Example 2
### Production of recombinant vector

The amphotropic virus used was MFG-lacZ. MFG
vectors are described in Dranoff et al. (1993) Proc. Natl.
Acad. Sci. USA 90:3539-3543. Amphotropic MFG-lacZ was
obtained as viral supernatant from Somatix Therapy
Corporation (Alameda, California). The viral titer
represented in Table 2 was determined by the supplier.

A VSV-G pseudotype virus was prepared using a cDNA
clone of the New Jersey isolate of the VSV-G envelope protein

as the source of the VSV-G gene.  Rose and Bergmann (1982)
Cell 30:753-762.  The VSV-G gene was subcloned by polymerase
chain reaction (PCR) on a Cetus GeneAmp 9600 machine using
100 ng plasmid; 10 ng each primer and 10 cycles of the
following:  30 sec 92°C, 30 sec 55°C, and 1 min 72°C.  The
following primers were used in PCR:
SEQ ID NO 1    5' GCGCGGGAATTCTGACACTATGAAGTGCCT 3'
SEQ ID NO 2    5' CCGGAGTCTAGAGTGCAGGATTTGAGTTAC 3'
Primer No. 1 was the 5' primer and generated an EcoRI site,
Primer No. 2 was the 3' primer and generated an XbaI site.
The resulting fragment was digested with EcoRI and XbaI and
cloned into plasmid pME-18S digested with EcoRI/XbaI to
generate plasmid pME-VSV-G.  pME-18S was obtained from DNAX;
plasmid pME-VSV-G is depicted in Figure 1.  Plasmid MFG-lacZ
is depicted in Figure 2.

The pseudotyped virus was prepared by transducing
the ANJOU cell line according to the method described by Pear
et al. (1993) Proc. Natl. Acad. Sci. USA 90:8392-8396.  ANJOU
cells used in transfection were maintained in Dulbecco's
modified Eagle's medium (DMEM) supplemented with 10% (v/v)
FCS, 2 mM l-glutamine, penicillin (100 U/ml), and
streptomycin (100 µg/ml) and grown at 37°C in 5% $CO_2$.  NIH3T3
cells were maintained in similar media but containing 10%
(v/v) calf serum instead of FCS.

ANJOU cells were plated at $10^7$/15 cm plate 18 hours
prior to transfection.  Standard calcium phosphate
transfection solution (Pear et al. (1993)) was prepared with
30 µg MFG-LacZ and 30 µg pME-VSV-G and added dropwise to
cells in media containing 25 µg/ml chloroquine to increase
transfection efficiency.  Six to 8 hours later the media was
changed to 10 ml fresh DMEM, cells were grown an additional
10 hours and the media changed again.  Viral supernatants
were collected 48 hours after transfection and either (1)
used to infect target cells, (2) frozen down at -20°C, or
(3) concentrated as discussed below.  ANJOU cells were fixed
in a 2% paraformaldehyde/0.2% glutaraldehyde solution and

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stained with X-Gal to determine the transfection efficiency.
Pear et al. (1993).   The virus obtained was designated VSV-G-
MFG-lacZ.

Concentration and titration of virus

Harvested supernatants from confluent ANJOU
producer cells were centrifuged to remove cellular debris at
200 rpm for 10 min, then subjected to ultracentrifugation in
a Beckman Model centrifuge in an SW28 rotor at 25,000 rpm at
4°C for 1.5 hours.   The pellet was resuspended overnight at
4°C in 300 ml TNE (50 mM Tris-HCl, pH 7.8, 130 mM NaCl, 1 mM
EDTA) and then frozen at -20°C.   Titered amounts of the
concentrated or the non-concentrated virus were used to
infect target NIH3T3 cells.

The viruses were titered on target cells plated in
3 ml of appropriate media in 6 cm plates at $2x10^5$ cells per
plate to achieve 60-73% confluence at the time of infection.
Eighteen to twenty-four hours after seeding, the media was
supplemented with 5 µg/ml polybrene and virus was added
immediately afterward.   Media was changed to 6 ml of fresh
media 8 hours after addition of virus to avoid polybrene
toxicity.   Forty-eight hours after infection, the media was
removed and cells were assayed for β-galactosidase activity
by X-gal or FACS-Gal according to the method described by
Nolan et al. (1988) Proc. Natl. Acad. Sci. USA 85:2603-260
for determination of viral titers.   The titration results are
shown in column 3 of Table 2.

Transductions were performed as described in
Example 3, using concentrated, frozen, virus preparations.

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## Example 3
### Transduction

1 x $10^5$ CD34⁺ (ABM) or 1 x $10^5$ CD34⁺Thy⁺Lin⁻ (MPB) viable cells obtained as described in Example 1, were suspended in 1 ml of freshly thawed retroviral supernatant with cytokines at the following concentrations: c-kit ligand (#mgen) 100 ng/ml; IL-3 (Sandoz) 25 ng/ml; IL-6 (Sandoz) 50 ng/ml. Protamine sulfate was added at a final concentration of 4 µg/ml. At 24 and 48 hours, supernatant was replaced with freshly thawed retroviral supernatant. Cytokines and protamine sulfate were added at the concentrations listed above. After 72 hours, cells were harvested and transduction frequency was determined.

Alternatively, 1 x $10^5$ CD34⁺ (ABM) or 1 x $10^5$ CD34⁺Thy-1⁺Lin⁻ (MPB) cells obtained as described in Example 1 were transduced as described above except that the cells were incubated with the retroviral supernatant for 16 hours without change of supernatant. The results of the 16 hour transduction are shown in Tables 2 and 3.

## Example 4
### Methylcellulose assay

In order to determine transduction frequency of the stem cells, the following experiment was performed. 5 x $10^3$ or 2.5 x $10^3$ cells from each transduction were added to 5 ml of methylcellulose (Stem Cell Technologies) containing the following cytokines: c-kit ligand 10 ng/ml; GM-CSF 25 ng/ml; G-CSF 25 ng/ml; IL-3 10 ng/ml; rhEPO 2 units/ml. 1.1 ml of the cell/cytokine methylcellulose mixture was plated onto four 3 cm gridded plates using a 5 ml syringe and 16.5 gauge needle, and the plates were placed in a 37°C incubator for 2 weeks.

After 14 days, 2-5 methylcellulose colonies were pooled into 200 µl PBS for PCR analysis. The cells were vortexed 5 seconds, centrifuged for 30 seconds at 10,000 rpm

in a microcentrifuge, and the PBS was removed by aspiration. 100 µl of Florence Lysing Buffer containing proteinase K (50 mM KCl, 20 mM Tris-HCl pH 9.0, 1.5 mM MgCl$_2$, 0.5% Tween 20, 0.5% NP40, 1 mg/ml proteinase K) was added to the cell pellet and vortexed to generate a cell lysate. The cell lysate was incubated 60 minutes in a 60°C water bath and subjected to flash spin. The cells were incubated 15 minutes in a 95°C heat block and subjected to flash spin. The lysates were stored at 4°C.

The lysates were amplified by PCR to determine the presence of the vector in the transduced cells. The PCR assay amplified a 539 bp fragment from MFG-lacZ. A 40 cycle amplification (50 µl total volume) in Perkin-Elmer 480 Cycler using 25 µl lysate and 100 ng each LZ 4927 and LZ 5466 (Keystone) Econopure primers was performed as follows: one cycle 95°C 6 min, 62°C 1 min 72°C 2 min; 4 cycles 95°C 2 min, 62°C 1 min, 72°C 2 min; 35 cycles 95°C 2 min, 62°C 1 min, 72°C 2 min; final extension 72°C 10 min. PCR products were visualized on ethidium bromide agarose gel and the results were verified by Southern Blot hybridization with a lacZ probe. As an alternative to detection by Southern blot hybridization, in some cases, a second, nested amplification was performed using a 30 cycle amplification in PE cycler using 5 µl of primary PCR product and 100 ng each of LZ4953 and LZ5425 (Keystone) Econopure primers.

The results obtained are presented in Table 2. Note that the transduction frequency represented in the final column represents a minimum transduction frequency since it assumes that, in a positive sample, only one colony of each pooled sample was positive. At higher transduction frequencies, the number presented is likely to be an underestimate of the actual transduction frequency.

Table 2
Summary of Transduction
Using Amphotropic and VSV-G Pseudotyped Retroviral Factors

| Tissue | Retroviral Vector | Viral Titer | Length of Transduction(hr) | Colonies Pooled/Sample | No. Samples | PCR Positive Samples | Transduction Frequency |
|---|---|---|---|---|---|---|---|
| CD34+Thy+Lin- (MPB) | MFG-lacZ | $5\text{-}10 \times 10^5$ | 72 | 5 | 36 | 10/36 | 10/180 (6%) |
| CD34+Thy+Lin- (MPB) | VSV-G-MFG-lacZ | $2 \times 10^6$ | 72 | 3 | 20 | 18/20 | $\geq 18/80$ ($\geq 30\%$) |
| Total CD34+ (ABM) | MFG-lacZ | $5\text{-}10 \times 10^5$ | 72 | 5 | 36 | 16/36 | $\geq 16/180$ ($\geq 9\%$) |
| Total CD34+ (ABM) | VSV-G-MFG-lacZ | $2 \times 10^6$ | 72 | 5 | 20 | 11/20 | 11/100 (11%) |
| CD34+Thy+Lin- (MPB) | MFG-lacZ | $5\text{-}10 \times 10^6$ | 16 | 5 | 36 | 0/36 | 0/180 (0%) |
| CD34+Thy+Lin- (MPB) | VSV-G-MFG-lacZ | $8 \times 10^5$ | 16 | 2 | 34 | 3/34 | 3/68 (4%) |
| Total CD34+ (ABM) | VSV-G-MFG-lacZ | $8 \times 10^5$ | 16 | 2 | 38 | 1/38 | 1/76 (1%) |

## TABLE 3

### Gene Transfer Into Hematopoietic Stem/progenitor Cells with VSV-G Pseudotype and Amphotropic Retroviruses: 16hr Transduction

| Tissue | Retroviral Vector | Viral Titer | Viable Cell Recovery (per 100K) | MeCell Colony Frequency (Per 10K) | MeCell Transduction Frequency |
|---|---|---|---|---|---|
| CD34+Thy+Lin- (MPB) | VSG-G-MFG-lacZ | $8 \times 10^5$ | 19K | 495 (1/20) | 4% (3/68) |
| CD34+Thy+Lin- (MPB) | MFG-lacZ | $5 \times 10^5$ | 51K | 577 (1/17) | 0% (0/180) |
| Total CD34+ (ABM) | VSV-G-MFG-lacZ | $8 \times 10^5$ | 22K | 2430 (1/4) | 1% (1/72) |

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The results presented above show that the VSV-G pseudotyped vector transduces CD34⁺Thy-1⁺Lin⁻ MPB stem cells with three to four fold greater efficiency than CD34⁺ ABM cells. Transductions were performed for either 72 hours (Table 2) or 16 hours (Tables 2 and 3). While transduction efficiency was higher with the longer transduction, the relative increase in transduction frequency was the same under both conditions. Under both transduction conditions, the VSV-G pseudotyped vector transduced MPB stem cells with much greater efficiency than the comparable amphotropic vector (on the order of five-fold in the 72 hour transduction; the 16 hour transduction cannot be compared since there were no transductions detected with the amphotropic vector). The observed differences are not due to patient variability or variation in the stem cell preparation since, in each case, the MPB samples from a single purification for the same patient were transduced with both the pseudotyped and amphotropic retroviral vectors. By comparison, the amphotropic vector transduced CD34⁺ ABM cells at a slightly higher frequency than CD34⁺Thy-1⁺Lin⁻ MPB cells, although this difference may not be statistically significant. Even with the amphotropic vector, it is likely that the stem cells are transduced at a higher frequency in the CD34⁺Thy-1⁺Lin⁻ population than in the CD34⁺ population.

In both the 16 and 72 hour transductions with the VSV-G pseudotyped vector, there appeared to be a greater loss in viable cells than in transductions with the amphotropic vector. The clonogenic potential of the recovered cells in methylcellulose, however, did not appear to be significantly affected. (Table 3) Preliminary observations suggest that much of the loss in viability is attributable to the buffer used in the VSV-G virus preparations, rather than to the VSV-G vector itself.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to
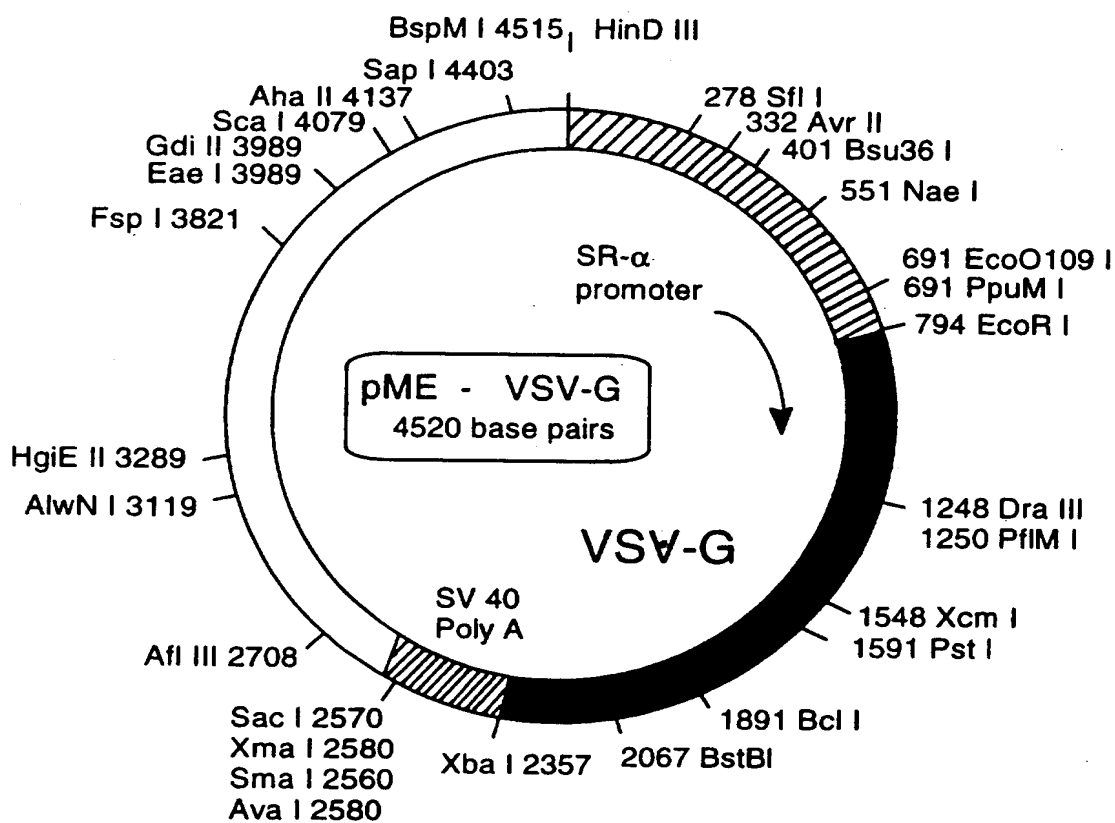
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those skilled in the art that certain changes and
modifications may be practiced.  It should be apparent that
one having ordinary skill in this art would be able to
surmise equivalents to the invention as described in the
claims which follow but which would be within the spirit of
the description above.  Those equivalents are to be included
within the scope of this invention.  Therefore, the
description and examples should not be construed as limiting
the scope of the invention, which is delineated by the
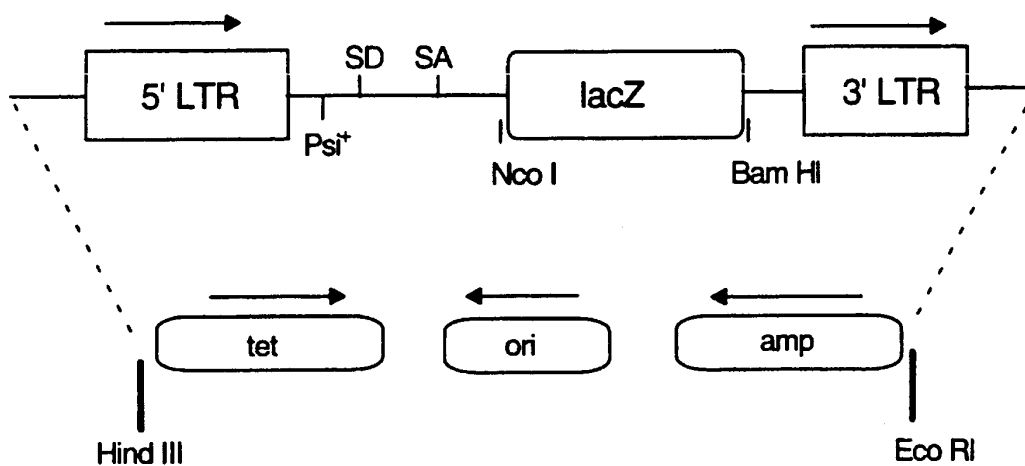appended claims.

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## Claims

1.    A method for genetically modifying
hematopoietic stem cells comprising:
        obtaining hematopoietic stem cells; and
        transducing the stem cells with a viral vector
bearing the vesicular stomatitis G protein.

2.    The method according to claim 1 wherein the
stem cells are CD34$^+$Thy-1$^+$Lin$^-$.

3.    The method according to claim 1 wherein the
stem cells are obtained from bone marrow, mobilized
peripheral blood or umbilical cord blood.

4.    The method according to claim 3 wherein the
stem cells are obtained from bone marrow or umbilical cord
blood and are CD34$^+$Thy-1$^+$Lin$^-$.

5.    The method according to claim 3 wherein the
stem cells are isolated from mobilized peripheral blood.

6.    The method according to claim 5 wherein the
stem cells are CD34$^+$Thy-1$^+$.

7.    The method according to claim 1 wherein the
viral vector is MFG.

8.    The method according to claim 1 wherein the
stem cells are isolated from mobilized peripheral blood and
the viral vector is MFG.

BspM I 4515 ┐ HinD III
Sap I 4403
Aha II 4137
Sca I 4079
Gdi II 3989
Eae I 3989
Fsp I 3821

278 Sfl I
332 Avr II
401 Bsu36 I
551 Nae I

691 EcoO109 I
691 PpuM I
794 EcoR I

SR-α
promoter

pME - VSV-G
4520 base pairs

VSV-G

1248 Dra III
1250 PflM I

HgiE II 3289
AlwN I 3119

1548 Xcm I
1591 Pst I

SV 40
Poly A

Afl III 2708

Sac I 2570
Xma I 2580
Sma I 2560
Ava I 2580

Xba I 2357

1891 Bcl I
2067 BstBI

1/2

SUBSTITUTE SHEET (RULE 26)

# MFG-lacZ

SUBSTITUTE SHEET (RULE 26)

International Application No

PCT/US 95/11892

## A. CLASSIFICATION OF SUBJECT MATTER
IPC 6    C12N15/87    C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6    C07K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,92 14829 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 3 September 1992 cited in the application see page 4, line 27 - page 6, line 23 see page 7, line 14 - page 8, line 26 see page 25, line 29 - page 26, line 2 see page 36, line 30 - page 37, line 29 --- | 1,3 |
| A | WO,A,88 08450 (FINLAYSON, BIRDWELL ET AL.) 3 November 1988 see page 1, line 1 - line 20 see page 5, line 5 - page 6, line 14 see page 23, line 23 - page 32, line 8; examples 6,7 --- | 1-8 |
| A | WO,A,93 18137 (SYSTEMIX, INC.) 16 September 1993 see page 33, line 1 - page 36, line 14 --- | 1-8 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 January 1996 | 29. 02. 96 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016 | Montero Lopez, B |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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